Jpn. J. Cancer Res. 80, 1179–1185, December 1989

# A Variant Schmidt-Ruppin Strain of Rous Sarcoma Virus with Increased Affinity for Mammalian Cells

Sadaaki Kawai, Makoto Nishizawa, Hideko Shinno-Kohno and Kazuko Shiroki

*Department of Tumor Virus Research, The Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108*

**SR-RSV-D(H), a variant virus with extremely high tropism for mammalian cells, was isolated by passage of the Schmidt-Ruppin strain of Rous sarcoma virus of subgroup D (SR-RSV-D) through hamster cells. This variant virus has acquired an altered envelope glycoprotein, encoded by the *env* gene, that has high affinity for receptors on the surface of mammalian cells. The variant virus transforms rat cells at about 100 times the efficiency of the parental virus, SR-RSV-D(S), as assayed by focus formation. Addition of amphotericin B (Fungizone) to the medium at a concentration of 0.2 μg/ml completely inhibited rat cell transformation by SR-RSV-D(H), possibly by blocking virus penetration into the cells, whereas the drug showed no inhibitory effect on transformation of chick embryo fibroblast (CEF) cells by the variant virus or on transformation of rat cells by the parental virus. The efficiency of transformation of rat cells by the variant virus was much less than its efficiency of transformation of CEF cells. Analysis of infection of rat cells suggested that the virus can infect rat cells as efficiently as CEF cells but that rat cells were not transformed by the virus as fully as CEF cells because of inefficiency of some post-penetrational step involved in viral gene expression. The finding that E1AY cells, rat cells expressing adenovirus E1A gene, were transformed by SR-RSV-D(H) as efficiently as CEF cells supports this conclusion and suggests that expression of the E1A gene in rat cells may overcome the defect in the transforming step(s) in rat cells.**

Key words: Rous sarcoma virus — Mammalian tropism — Envelope glycoprotein — Cell fusion — Adenovirus E1A gene

Avian leukosis-sarcoma viruses are classified into subgroups A through E according to their host ranges, which are determined by the *env* gene product (envelope glycoprotein) expressed on the virus particles. All avian leukosis-sarcoma viruses can infect and grow well in chicken cells except for genetically resistant cells (for example, C/A-type and C/B-type chicken cells are resistant to subgroup A and B viruses, respectively, and C/O-type cells are susceptible to viruses of all subgroups). Many strains of Rous sarcoma virus (RSV) with different host ranges have emerged from the original isolate from chicken tumor No. 1.[1] Alteration of the host range is known to occur through genetic recombination with avian leukosis viruses of different subgroups[2, 3] and mutations in the *env* gene[4, 5] during long-term passages in chickens or tissue cultures. RSV strains of subgroups C and D are known to be able to transform mammalian cells besides chicken cells, although their efficiencies of transformation of mammalian cells are much lower, partly because the *env* gene products have much lower affinity for receptors on mammalian cells.[6-8] These mammalian tropic RSV strains have the unique property of being able to transform mammalian cells but not grow in them.[9] That is, RSV-transformed mammalian cells do not produce infectious RSV. However, when these cells are fused with chicken cells, infectious RSV particles can be rescued, suggesting that mammalian cells lack some cellular factor(s) required for a certain step(s) of RSV replication, and that this factor(s) can be supplied from chicken cells by cell fusion.[10-12] Because of these unique characteristics, this avian retrovirus with affinity for mammalian cells should provide a useful vector system for introducing exogenous genes into mammalian cells and studying the functions of the genes in these cells without interference by effects caused by the spread of infectious virus in the system. The present paper describes studies on a variant RSV with very high affinity for mammalian cell receptors, which should be suitable for development of this potentially useful vector system.

## MATERIALS AND METHODS

**Cells and viruses** Chicken embryo fibroblast (CEF) cells were prepared from fertile eggs (free from endogenous virus) kindly supplied by the Nippon Institute of Biological Science, Tokyo. CEF cells were grown in Eagle's minimal essential medium (Nissui Seiyaku, Tokyo) supplemented with 10% tryptose phosphate broth and 5% calf serum. A rat cell line, 3Y1,[13] and E1AY cells,[14] 3Y1 cells expressing the E1A gene of human adenovirus type 12, were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Standard strain of Schmidt-Ruppin Rous sarcoma virus of subgroup D (SR-

RSV-D(S)), the Prague strain of RSV (PR-RSV) and B77 ASV were obtained from Dr. H. Hanafusa. Fungizone (Squibb) was used as amphotericin B in this study.
**Focus assay** Focus titers on CEF and rat 3Y1 cells were assayed as described previously.[15, 16]
**Cell fusion** Samples of $2\times10^5$ 3Y1 cells of each clone, which had been cloned after infection with RSV, and $1\times10^6$ CEF cells were mixed and seeded in 60 mm dishes. After incubation in a $CO_2$ incubator for 24 h they were treated with 50% polyethylene glycol as described previously.[16-18] Culture fluids harvested on day 7 were tested on CEF cells for production of infectious RSV.
**Isolation of transformation-defective (td) mutants of SR-RSV-D(H)** A sample of $1\times10^7$ CEF cells suspended in 100 ml of medium were inoculated with about 36 FFUs of SR-RSV-D(H) and seeded into 96 wells. After incubation for 7 days at 41°C, each well was examined by microscopy for the presence of transformed cells. Culture fluids from 70 wells in which no transformed cells were detected were harvested, and inoculated separately into dishes (35 mm) containing $3\times10^5$ fresh CEF cells. The cultures were incubated for 6 days and then transferred to 60 mm dishes. After incubation for 4 days, the culture fluids were harvested and stored at $-70$°C. The remaining cells were again transferred and infected with $1\times10^4$ FFUs of SR-RSV-D(H). Cell transformation was checked 5 days later and two of the 70 cultures were found not to be transformed by SR-RSV-D(H) because of virus interference. Transformation-defective mutants recovered from frozen culture fluids were grown fully in tissue culture to prepare virus stocks.

## RESULTS

**Attempt to isolate an RSV variant with high affinity for mammalian cells** The plating efficiency of a mammalian-tropic avian sarcoma virus on rat cells has been reported to be increased by passage through a rat cell line due to some relatively stable genetic change of the viral genome. The mechanism of this genetic change is unknown, but has been suggested to be due to genetic recombination with some cellular sequence in the rat cell.[19]

In this work, we first examined the plating efficiencies on rat cells of several mammalian-tropic strains of RSV maintained in our laboratory and found that their transforming titers on 3Y1 cells were very low, usually between about $10^{-4}$ and $10^{-6}$ of those on CEF cells. None of these viruses appeared to be suitable for use in obtaining a virus vector for mammalian cells. Therefore, based on the phenomenon described above, we tried to obtain a variant virus with higher ability to infect mammalian cells by passage through hamster cells. CEF cells (about $6\times10^6$ cells) fully transformed with the standard strain of SR-RSV-D (SR-RSV-D(S)), which had been passaged serially for a long time on CEF in tissue culture, were co-cultured with freshly prepared hamster embryo cells ($1\times10^6$ cells) for 4 days and inoculated subcutaneously into 4 adult hamsters. Four weeks later, a tumor induced at the injection site was excised and trypsinized. For rescue of infectious SR-RSV-D virus, the tumor cells were co-cultured with CEF cells in the presence of UV-inactivated Sendai virus, which facilitates cell fusion. The culture fluid harvested on day 10 was inoculated onto CEF cells and infected cells were grown in soft-agar suspension cultures for 2 weeks. One of resulting transformed cell colonies was isolated and grown with growth medium. When the cells were fully grown, the culture fluids were harvested, pooled and stored at $-75$°C as a cloned virus stock until use. The focus-forming titer of the original virus stock was assayed on CEF and rat 3Y1 cells. The results, shown in Table I, indicated that the SR-RSV-D(H) recovered from transformed hamster cells had acquired higher specific activity for transforming rat cells than the parental SR-RSV-D(S), PR-RSV-C and B77 viruses. It should be noted, however, that the efficiency of even SR-RSV-D(H) for transforming rat cells was still only about one-sixtieth of that for transforming CEF cells. Similar results were obtained when these viruses were assayed on mouse NIH3T3 and Balb3T3 cells (data not shown). Furthermore, when freshly prepared mouse primary cells were infected with SR-RSV-D(H), cell transformation was observed after several transfers while SR-RSV-D(S), B77 and PR-RSV-C viruses did not transform the primary mouse cells. These findings indicated that SR-RSV-D(H) virus has high plating ability on not only rat cells, but also mouse cells.

Table I. Comparison of Focus-forming Activities of SR-RSV-D Strains

| Virus | Titer[a] in | | Ratio | |
|---|---|---|---|---|
| | 3Y1 | CEF | 3Y1/CEF | Relative activity in 3Y1[b] |
| SR-RSV-D(H) | $2.4\times10^3$ | $1.5\times10^5$ | 0.016 | 133 |
| SR-RSV-D(S) | $2.0\times10^2$ | $1.7\times10^6$ | 0.00012 | 1 |
| B77 | 10 | $8.0\times10^6$ | $1.2\times10^{-6}$ | 0.01 |
| PR-RSV-C | 5 | $1.0\times10^6$ | $5.0\times10^{-6}$ | 0.04 |

*a*) FFU/ml.
*b*) Ratio relative to SR-RSV-D(S).

**Properties of SR-RSV-D(H) virus** Increased ability of SR-RSV-D(H) to transform rat cells may be explained either by more efficient penetration of the virus particles into the rat cells or enhanced expression of the virus genes in rat cells. In the former case, the virus envelope glycoprotein encoded by the *env* gene may be altered. To

Table II. Subgroup Specificities of SR-RSV-D(S) and SR-RSV-D(H) Viruses

| Virus | Titer[a] in | | Ratio |
|---|---|---|---|
| | C/O | C/BE | |
| SR-RSV-D(H) | $3.3\times10^4$ | $2.0\times10^4$ | 0.61 |
| SR-RSV-D(S) | $3.1\times10^5$ | $1.9\times10^3$ | 0.0061 |
| SR-RSV-B | $1.0\times10^7$ | $<10$ | $<1.0\times10^{-5}$ |

*a*) FFU/ml.

Table III. Effect of Polybrene on Virus Adsorption

| Virus | Polybrene | | Ratio |
|---|---|---|---|
| | − | + | (−/+) |
| SR-RSV-D(H) | $2.5\times10^5$ | $2.5\times10^5$ | 1.0 |
| SR-RSV-D(S) | $2.0\times10^5$ | $3.7\times10^6$ | 0.055 |

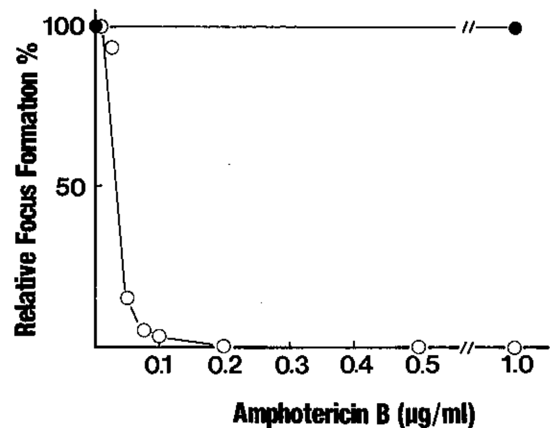


Fig. 1. Dose-dependence of effect of amphotericin B on transformation of rat 3Y1 cells. Samples of $1\times10^5$ 3Y1 cells were seeded in 60 mm plastic dishes with growth medium containing the indicated concentrations of amphotericin B and were inoculated, 12 h later, with 0.1 ml of SR-RSV-D(S) (●) or SR-RSV-D(H) (○). Culture fluids were renewed every 5 days and foci were counted after incubation for 3 weeks.

see if there was a change in host range specified by the envelope glycoprotein, we examined the abilities of the SR-RSV-D(H) and SR-RSV-D(S) viruses to infect C/O chicken cells, which are fully susceptible to all avian viruses of all subgroups and C/BE chicken cells, which are highly resistant to infection by viruses of subgroups B, D, and E. The results in Table II show that SR-RSV-D(H) was much more infectious than SR-RSV-D(S) to C/BE cells. Since the susceptibility of these cells to viruses of different subgroups are known to be determined by the presence or absence of specific receptors on the cells, this result indicates that the properties of the *env* gene product of SR-RSV-D(H) virus are different from that of SR-RSV-D(S). Addition of polycations such as Polybrene to assay cultures is known to increase virus titers by enhancing virus adsorption on cells, depending on the electrostatic properties of the viral surface.[20] Therefore, we next tested the effect of Polybrene on the infectivities of these SR-RSV-D viruses. Polybrene had no effect on focus formation by SR-RSV-D(H) virus, but greatly enhanced focus formation by SR-RSV-D(S) (Table III). These findings also suggest that the properties of the envelopes of these two virus are different.

**Amphotericin B (Fungizone) inhibits transformation of rat cells by SR-RSV-D(H) but not by SR-RSV-D(S)** During the course of this study we found that amphotericin B (Fungizone), an antifungal substance that is known to form a complex with sterol and steroid on the cell membrane,[21] strongly inhibited focus formation in rat cells by SR-RSV-D(H) at about the concentration (1 μg/ml) usually employed as a fungicide, but that it did not inhibit cell transformation by SR-RSV-D(S) virus. As can be seen in Fig. 1, the inhibition showed a sharp dose response; focus formation by SR-RSV-D(H) was completely abolished by amphotericin B at concentrations of more than 0.2 μg/ml, but was not affected by the drug at concentrations of less than 0.02 μg/ml, and the concentration for 50% inhibition was about 0.035 μg/ml. No inhibitory effect was observed when rat cells were inoculated with SR-RSV-D(H) in the absence of the drug and then, after 12 h incubation for virus adsorption, the culture fluid was replaced by medium containing amphotericin B. The drug did not inhibit focus formation of SR-RSV-D(H) on CEF cells, excluding the possibility that it inactivates the virus directly. These findings together suggest that the drug affected a step of virus adsorption onto rat cells or the penetration of SR-RSV-D(H) into rat cells. The specific inhibitory effect of the drug on the transforming ability of SR-RSV-D(H) also suggests that the *env* gene-encoded envelope glycoprotein of this virus has some peculiar properties.

**Transformation-defective mutants isolated from SR-RSV-D(H) virus** Transformation-defective mutants, in which the whole sequence of the *src* gene is deleted, are frequently segregated from replication-competent RSV viruses during virus replication.[22-24] As described in the "Materials and Methods," we isolated transformation-defective mutants from a stock preparation of SR-RSV-D(H) virus to confirm that this high tropism for mammalian cells was due to change in the *env* gene of the SR-RSV-D(H) virus.

Table IV. Plating Efficiencies of B-RSV and NY8 Viruses Coated with td Mutant Isolated from SR-RSV-D(H)

| Virus | Titer[a] in | | | Ratio |
|---|---|---|---|---|
| | CEF | 3Y1 | | [3Y1(−)/CEF(−)] |
| | (Amph.)[b] (−) | (−) | (+) | |
| B-RSV (tdSR(H)) | $2.1\times10^4$ | $3.5\times10^3$ | <1 | 0.17 |
| B-RSV (RAV-50) | $7.0\times10^4$ | $3.0\times10^1$ | $3.0\times10^1$ | 0.00042 |
| NY8 (tdSR(H)) | $1.0\times10^4$ | $5.0\times10^1$ | <1 | 0.005 |
| NY8 (RAV-50) | $1.0\times10^5$ | <1 | <1 | <0.00001 |

*a*) FFU/ml.
*b*) (+) and (−) indicate the presence or absence of amphotericin B at the time of infection.

Cultures of CEF cells transformed by the Bryan high-titer strain of RSV (BH-RSV) and NY8, an *env*-deficient mutant of SR-RSV-A, which produce non-infectious virus particles deficient in the *env* gene product were prepared as described previously[25] and superinfected with td mutants of SR-RSV-D(H) and RAV-50 to obtain pseudoviruses, BH-RSV(tdSR(H)), BH-RSV(RAV-50), NY8(tdSR(H)), and NY(RAV-50), which are made infectious by being supplied with the *env* gene product from the td mutants and RAV-50.

For determination of the affinities of these pseudoviruses for mammalian cells, the viruses were assayed for focus formation on CEF and rat 3Y1 cells. As can be seen in Table IV, BH-RSV(tdSR(H)) virus showed much higher plating efficiency than BH-RSV(RAV-50) virus on rat cells and like SR-RSV-D(H) virus, BH-RSV(tdSR(H)) did not induce transformation of rat cells in the presence of amphotericin B, whereas BH-RSV(RAV-50) did, clearly indicating that the high affinity of SR-RSV-D(H) for mammalian cells is due to its *env* gene product. However, it was interesting that BH-RSV(tdSR(H)) had much higher transforming activity than NY8(tdSR(H)) in rat cells. The different plating efficiencies of these RSV viruses on rat cells suggest that rat cell transformation by RSV viruses may also depend upon some other factor(s) than the *env* gene. Both NY8 RSV and BH-RSV lack the *env* gene, but their genomic structures are not identical, and their *src* genes contain different mutations. Therefore, with these two viruses the expressions of the *src* gene and the transforming activities of the *src* gene products in rat cells may be different. We have not yet studied these interesting possibilities.

**Greatly increased penetrating efficiency of SR-RSV-D(H)** The findings described above showed that SR-RSV-D(H) has envelope glycoprotein wirh very high affinity for rat cell receptors. On the other hand, cell transformation by RSV viruses also depends on the extent of *src* gene expression in infected cells; rat cells infected with SR-RSV-D(H), in which the level of expression of the *src* gene product is high, are transformed and form foci, whereas on similar infection those that do not produce a sufficient amount of the *src* gene product cannot be recognized as transformed cell foci. Hence, focus formation does not reflect the efficiency of infection of cells by RSV virus. Therefore, the following experiment was carried out to determine the efficiency of SR-RSV-D(H) incorporation into rat cells through the interaction between the *env* gene products and cell receptors. A sample of $3\times10^5$ rat cells was inoculated with 1.0 ml of a SR-RSV-D(H) virus stock (containing $1.3\times10^5$ FFU of virus on CEF or $6.0\times10^3$ FFU on 3Y1 cells) and replated after 18 h at 30, 90, and 120 cells/plate. Rat cells similarly infected with a SR-RSV-D(S) virus stock were also plated in the same manner. Three weeks later, the cell colonies formed were counted and each colony was examined for the presence of transformed cells. As shown in Table V, about one-third of the colonies of SR-RSV-D(H)-infected cells were found to contain transformed cells, whereas none of the colonies of SR-RSV-D(S)-infected cells contained transformed cells. This result was surprising because the ratio of colonies containing transformed cells to normal colonies in SR-RSV-D(H)-infected cultures was much higher than that estimated from the focus-forming titer of the virus on rat cells. However, almost all colonies containing transformed cells were composed of mixtures of normal flat cells and round or fusiform, transformed cells, and very few colonies consisted entirely of transformed cells. This may indicate that full transformation of rat cells after SR-RSV-D(H) infection is very inefficient, and that focus assay may detect only this type of cell transformation. Furthermore, some colonies consisting only of apparently normal flat cells may have been infected but did not express the *src* gene sufficiently to induce the transformed state. To examine this possibility, we fused cells derived from colonies consisting entirely of apparently normal, flat cells with CEF cells and tested the

Table V. Efficiency of Infection of 3Y1 Cells with SR-RSV-D(Hm) Virus

| Virus | No. of cells seeded/plate | No. of foci (transformed/total) | | Ratio | No. of flat cells foci | Total ratio |
|---|---|---|---|---|---|---|
| | | individual plates | total | | virus rescuable / total | |
| SR-RSV-D(H) | 30 | 1/4, 1/3, 1/5, 2/5, 5/11 | 10/28 | 0.29 | 5/27 (0.18) | 0.41 |
| | 90 | 9/11, 9/34, 4/15, 6/22 | 28/95 | | | |
| | 120 | 20/65, 15/51, 21/84, 15/55 | 71/255 | | | |
| SR-RSV-D(S) | 30 | 0/8, 0/10, 0/11, 0/10, 0/8, 0/12 | 0/59 | 0.005 | 0/15 (0) | 0.005 |
| | 90 | 0/22, 0/24, 0/16, 0/19, 0/26, 0/16 | 0/123 | | | |

3Y1 cells ($3\times10^5$) were infected with 1.0 ml of virus stock and replated after 18 h at the cell numbers indicated. Titers of virus stocks: SR-RSV-D(H) $1.3\times10^5$ FFU/ml (on CEF), $6.0\times10^3$ FFU/ml (on 3Y1 cells); SR-RSV-D(S) $7.0\times10^5$ FFU/ml (on CEF), 20 FFU/ml (on 3Y1 cells).

Table VI. Transforming Titer of SR-RSV-D(H) on Rat E1AY Cells Expressing Adenovirus E1A Gene

| Virus | Amph.[b] | Titers[a] on | | Ratio | |
|---|---|---|---|---|---|
| | | CEF | E1AY | E1AY/CEF | Amph.(+/−) |
| SR-RSV-D(H) | − | $4.5\times10^5$ | $4.3\times10^5$ | 0.96 | |
| | + | | $4.6\times10^4$ | | 0.1 |
| SR-RSV-D(S) | − | $4.0\times10^6$ | $3.0\times10^3$ | 0.00075 | |
| | + | | $2.8\times10^3$ | | 0.93 |

*a*) FFU/ml.
*b*) Amphotericin B.

fused cells for production of infectious SR-RSV-D(H) virus. As can be seen in Table V, no virus was rescued from apparently normal colonies derived from cultures inoculated with SR-RSV-D(S), but SR-RSV-D(H) virus could be rescued from about 20% of similar, apparently normal cell colonies by fusion of the cells with CEF cells, indicating that SR-RSV-D(H) virus incorporated into these cells was present in a stable form but did not induce morphological change of the infected cells. These results indicated that the overall infection rate of rat cells was as high as 41%. This means that the number of virus particles ($1.2\times10^5$) taken up by rat cells was almost equivalent to the titer of the virus assayed on CEF cells. This finding indicates that the envelope glycoprotein of SR-RSV-D(H) has almost the same affinity for rat cell receptors as for chicken cell receptors.

**Efficient transformation by SR-RSV-D(H) of rat cells expressing adenovirus E1A gene** During the study we incidentally found that E1AY cells, rat 3Y1 cells that express the adenovirus early gene, E1A, were transformed morphologically as efficiently as CEF by SR-RSV-D(H) virus. E1AY cells are morphologically different from the parental 3Y1 cells because they are partially transformed by E1A gene expression. However, since they are flat and hexagonal when fully grown and show contact-inhibition in culture, transformed foci induced by SR-RSV-D(H) which are composed of characteristic round and piled-up cells were very easy to detect. The transforming activities of SR-RSV(H) and SR-RSV-D(S) were assayed on E1AY rat cells and CEF cells in the presence or absence of amphotericin B (Table VI). Although E1AY cells were refractory to transformation by SR-RSV-D(S) virus, their susceptibility to SR-RSV-D(H) virus was as high as that of CEF cells. These results suggest that the difference between the transforming activities of these viruses on E1AY cells may directly reflect the affinities of the *env* gene-encoded glycoproteins of these viruses for receptors on rat cells. Much lower sensitivity to amphotericin B of E1AY cells compared with that of 3Y1 cells may be explained by conformational change of the cell membrane of partially transformed E1AY cells. On the E1AY cell membrane, sterol and steroid may be arranged so that amphotericin B can not efficiently interact with them.

Studies are required on whether expression of the *src* gene is increased in these E1A cells compared with that in the parental 3Y1 cells or whether the threshold level of the *src* gene product for cell transformation is lowered in

the rat cells expressing the E1A gene. The establishment of the E1AY cell line and a more detailed study of transformation of E1AY cells by SR-RSV-D(H) will be described elsewhere.

## DISCUSSION

In this work we isolated a variant of SR-RSV-D with ability to infect mammalian cells, by passage of the virus through hamster cells. Analyses of this variant virus (SR-RSV-D(H)) showed that it has an altered envelope glycoprotein encoded by the *env* gene. This genetic change of the *env* gene may have resulted from genetic interaction with hamster cells. However, as avian sarcoma viruses are known to have a high mutation rate,[4, 5] the possibility that a spontaneous mutant was present in the virus stock and was selected during passage in hamster cells seems more likely. Conversely, when SR-RSV-D(H) was grown repeatedly in CEF cells, its titer on CEF cells gradually increased and its activity to transform rat cells decreased (data not shown). This phenomenon could also be explained in either of the above two ways. A comparison of the nucleotide sequences of the *env* gene of the parental virus with those of the virus infecting mammalian cells, and CEF-adapted viruses would provide information on the nature of the host range alteration.

Retrovirus can be used as an efficient vector to introduce exogenous genes into chromosomes for study of gene function and for gene therapy. However, replication of viruses in infected cells would cause severe problems, particularly when virus vectors are used for gene therapy. For avoiding such problems, helper virus-free retrovirus vector systems have been established based on amphotropic murine leukemia virus DNA[26] However, completely helper virus-free preparations have been very difficult to obtain: if virus preparations contain even a single infectious helper virus, the helper virus would replicate in treated cells and spread throughout cultures or animal bodies. Various attempts have been made to improve the vector systems in this respect.[27–30] In contrast to these vector systems based on amphotropic murine leukemia virus, avian retroviruses would provide alternative safer vector systems for mammalian cells, because avian retroviruses cannot replicate and, therefore, cannot spread in mammalian cells. A disadvantage of avian retroviruses is that they cannot be incorporated into mammalian cells with sufficient efficiency, mainly because of the low affinity of their viral envelope glycoproteins to mammalian cell surface receptors. However, the variant of SR-RSV-D that we isolated and characterized in this study can be incorporated into rat cells, and possibly also into other mammalian cells, at almost the same rate as into CEF cells, and so this disadvantage could be overcome by development of avian retrovirus vector systems utilizing the variant SR-RSV-D with this particular character. There may be some other problems in the use of an avian retrovirus vector system besides low affinity for mammalian cell receptors. For example, while SR-RSV-D(H) can transform E1AY cells as efficiently as CEF cells, the virus cannot transform the parental 3Y1 rat cells efficiently. This is possibly because of the low expression of the *src* gene in normal rat cells. The low expression of the *src* gene might be due to the weak promoter activity of RSV, depending on the site of integration of the RSV genome or an aberrant splicing mechanism of the *src* gene in un-natural host cells. Therefore, more detailed analyses of the expression of viral genes of RSV in mammalian cells is necessary as the next step in development of a useful avian retrovirus vector using SR-RSV-D(H).

## ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

*(Received July 7, 1989/Accepted September 28, 1989)*

## REFERENCES

1) Rous, P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J. Exp. Med.*, **13**, 397–411 (1911).
2) Kawai, S. and Hanafusa, H. Genetic recombination with avian tumor virus. *Virology*, **49**, 37–44 (1972).
3) Vogt, P. K. Genetically stable reassortment of markers during mixed infection with avian tumor viruses. *Virology*, **46**, 947–952 (1971).
4) Zarling, D. A. and Temin, H. M. High spontaneous mutation rate of an avian sarcoma virus. *J. Virol.*, **17**, 74–84 (1976).
5) Zarling, D. A., Mosser, A. G. and Temin, H. M. Spontaneous mutations affecting the host range of the B77 strain of avian sarcoma virus involve type-specific changes in the virion envelope antigen. *J. Virol.*, **21**, 105–112 (1977).
6) Duff, R. G. and Vogt, P. K. Characteristics of two new avian tumor virus subgroups. *Virology*, **39**, 18–30 (1969).
7) Hanafusa, H. and Hanafusa, T. Determining factor in the capacity of Rous sarcoma virus to induce tumors in mammals. *Proc. Natl. Acad. Sci. USA*, **55**, 532–538 (1966).
8) Boettiger, D., Love, D. N. and Weiss, R. A. Virus envelope markers in mammalian tropism of avian RNA

tumor viruses. *J. Virol.*, **15**, 108–114 (1975).
9) Svoboda, J. and Hlozanek, I. Role of cell association in virus infection and virus rescue. *Adv. Cancer Res.*, **13**, 217–269 (1970).
10) Svoboda, J., Machala, O. and Hlozanek, I. Influence of Sendai virus on RSV formation in mixed culture of virogenic mammalian cells and chicken fibroblasts. *Folia Biol.*, **13**, 155–157 (1967).
11) Shevliaghyn, V. J., Biryulina, T. I., Tikhonova, Z. N. and Karazas, N. V. Activation of Rous virus in the transplanted golden hamster tumor with the aid of artificial heterokaryon formation. *Int. J. Cancer*, **4**, 42–46 (1969).
12) Yamaguchi, N., Takeuchi, M. and Yamamoto, T. Rous sarcoma virus production in mixed cultures of mammalian Rous sarcoma cells and chick embryo cells. *Int. J. Cancer*, **4**, 678–689 (1969).
13) Kimura, G., Itagaki, A. and Sammers, J. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. *Int. J. Cancer*, **15**, 694–706 (1975).
14) Shiroki, K., Saito, I., Maruyama, K., Fukui, Y., Imatani, Y., Oda, K. and Shimojo, H. Expression of adenovirus Type 12 early region 1 in KB cells transformed by recombinants containing the gene. *J. Virol.*, **45**, 1074–1082 (1983).
15) Rubin, H. A virus in chick embryo which induces resistance to *in vitro* infection with Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA*, **46**, 1105–1119 (1960).
16) Kawai, S. Transformation of rat cells by fusion-infection with Rous sarcoma virus. *J. Virol.*, **34**, 772–776 (1980).
17) Pontecorvo, G. Production of mammalian cell hybrids by means of polyethylene glycol treatment. *Somat. Cell Genet.*, **1**, 397–400 (1975).
18) Steimer, K. S. and Boettiger, D. Complementation rescue of Rous sarcoma virus from transformed mammalian cells by polyethylene glycol-mediated cell fusion. *J. Virol.*, **23**, 133–141 (1977).
19) Alterner, C. and Temin, H. M. Carcinogenesis by RNA sarcoma viruses XII. A quantitative study of infection of rat cells *in vitro* by avian sarcoma viruses. *Virology*, **40**, 118–134 (1970).
20) Toyoshima, K. and Vogt, P. K. Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology*, **38**, 414–426 (1969).
21) Lampen, J. O. Amphotericin B and other polyenic antifungal antibiotics. *Am. J. Clin. Pathol.*, **52**, 138–146 (1969).
22) Martin, G. S. and Vogt, P. K. The $\alpha$ subunit in the RNA of transforming avian tumor viruses. I. Occurrence in different virus strains. II Spontaneous loss resulting in non-transforming variants. *Virology*, **47**, 494–497 (1972).
23) Vogt, P. K. Spontaneous segregation of non-transforming viruses from cloned sarcoma viruses. *Virology*, **46**, 939–946 (1971).
24) Kawai, S., Metroka, C. E. and Hanafusa, H. Complementation of functions required for cell transformation by double infection with RSV mutants. *Virology*, **49**, 302–304 (1972).
25) Kawai, S. and Hanafusa, H. Isolation of defective mutant of avian sarcoma virus. *Proc. Natl. Acad. Sci. USA*, **70**, 3493–3497 (1973).
26) Mann, R., Mulligan, R. C. and Baltimore, D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell*, **33**, 153–159 (1983).
27) Basselman, R. A., Hsu, R., Bruszewski, Hu, S., Martin, F. and Nicolson, M. Replication-defective chimeric helper proviruses aand factors affecting generation of competent virus: expression of Moloney murine leukemia virus structural genes via the metallothionein promoter. *Mol. Cell. Biol.*, **7**, 1797–1806 (1987).
28) Hock, R. A. and Miller, A. D. Retrovirus-mediated transfer and expression of drug-resistant genes in human haematopoietic progenitor cells. *Nature*, **320**, 275–277 (1986).
29) Miller, A. D. and Buttimore, C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.*, **6**, 2895–2902 (1986).
30) Markowitz, D., Goff, S. and Bank, A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.*, **62**, 1120–1124 (1988).